ESTABLISHMENT AND VALIDATION OF *MYCOBACTERIUM FORTUITUM* MODEL FOR *MYCOBACTERIUM TUBERCULOSIS* PERSISTENT INFECTION

SYNOPSIS

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ESTABLISHMENT AND VALIDATION OF *MYCOBACTERIUM FORTUITUM* MODEL FOR *MYCOBACTERIUM TUBERCULOSIS* PERSISTENT INFECTION

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Abstract

Persistent form of tuberculosis remains major hurdle in absolute and effective control of the disease. As processes and mechanisms leading to development of persistent infection may take long time to manifest in host, thus, various *in vitro* models of *Mycobacterium tuberculosis* persistent infection have been developed. Study of model systems using *M. tuberculosis* are associated with constrains in terms of cost (establishment and maintenance of BSL3) as well as screening period (longer generation time). Aim of the present study was to develop a rapidly growing mycobacteria based model mimicking *M. tuberculosis* persistent infection which may offer advantages in terms of cost effectiveness and comparatively faster screening time for potential drugs and drug targets. *Mycobacterium fortuitum* was selected on the basis of shorter generation time, ease of handling, non-requirement for biosafety level-3 and closer homology with *M. tuberculosis* genome sequence. On subjecting to physiological stress conditions encountered by persistent *M. tuberculosis*, *M. fortuitum* showed constant CFU for extended periods of time, the most important characteristic feature of a persistent infection. Further validation of the organism also confirmed that it exhibited all physiological features of a persistent infection and up regulation of genes involved in persistence. Identification of five novel gene sequences of *M. fortuitum* was accomplished as homologues of *M. tuberculosis* genes involved in persistence, and submitted to GenBank database. Upregulation of these *M. fortuitum* homologues studied by real-time PCR predicted their involvement in persistence of *M. fortuitum.* An antisense knockout study of three of these genes was done, which further confirmed their role in *M. fortuitum* persistence. Thus, our studies suggest a conserved mechanism of persistence in *M. fortuitum* and *M. tuberculosis.* Studies related to behaviour of *M. fortuitum* under acid-induced stress indicate that acid-induced stress may simulate the conditions of persistent form of bacilli, thus, this condition can also be exploited to develop model for *M. tuberculosis* persistent infection.

Introduction

Tuberculosis (TB) is an infectious disease caused by *M. tuberculosis*. TB is one of the leading causes of death in the world per year and declared as global emergency by WHO (World Health Organisation) in 1993. *M. tuberculosis* remains one of the world's most lethal and resilient organisms accounting for 9.6 million infections and 1.5 million deaths in 2015 alone [1]. Mortality due to TB remains a global problem and the scenario is further worsened due to increasing number of AIDS (Acquired Immune Deficiency Syndrome) cases.

Genus *Mycobacterium* is composed of slightly curved or straight rods that may sometimes branch or form filaments, resembling fungal mycelium [2]. In liquid cultures they form mold like pellicle and hence named '*Mycobacteria*' meaning fungus like bacteria. The bacilli do not stain readily but once stained resist decolorization with mineral acids. Hence they are also known as 'acid fast bacilli' (AFB). Lepra bacilli were the first member of the genus to be identified by Hansen (1873). The second group comprised of mammalian tubercle bacilli as causative agent of tuberculosis, isolated by Robert Koch (1882). The third group comprises of mixed group isolated from diverse sources like birds, cold blooded animals, skin lesions, cow dung and environment (soil and water). This group was initially termed as 'atypical mycobacteria' because members of the group do not form characteristic structures called 'tubercle' [3, 4].

Latent TB infection

TB infection in humans can be presented in two different forms 'active' and 'latent'. Active TB involves population displaying characteristic symptoms of the disease and harbouring proliferating *M. tuberculosis* bacteria. Latent infection is characterized by asymptomatic state of the population, acting as a potential reservoir of the infection. One third of the human population is predicted to carry *M. tuberculosis* latent infection [5, 6]. There are multiple factors that regulate the course and outcomes of infection by *M. tuberculosis*, which involve a complex interplay between the immune system of the host and survival strategies employed by the bacilli [7]. The success of *M. tuberculosis* as a pathogen is due to its ability to persist for long periods of time in the face of immunity and chemotherapy [8] without producing specific symptoms of the disease [9]. On exposure to *M. tuberculosis*, less than 10% of the infected individuals develop an active form (symptoms) of the disease, while in the rest, the infection is present in an asymptomatic, persistent or latent form [10]. Such latent carriers

may have upto 10% risk of reactivation (per life time) to active TB infection state and this risk ascends with factors like immunosuppression, age, HIV infection, diabetes etc [11]. In addition to high morbidity and mortality associated with TB infections; very less is understood with respect to critical pathogen driven factors and host immune response involved in latent infection and reactivation [12].

Latency is a condition of equilibrium between the host and bacilli where, in response to the bacterial pathogenesis the host mounts a strong immune response leading to the formation of 'granulomatous structures' surrounding a small population of dormant bacterial cells [7]. Granuloma formation is an imperative mechanism for control of bacterial dissemination and is believed to be the seat of persistent bacilli. Inside granuloma, bacilli are exposed to multiple stress conditions of oxygen deficit (hypoxia), nutrient starvation and acidic pH. Different stress conditions encountered by the latent bacilli inside such granulomatous structures have been used to mimic *M. tuberculosis* latent infection under *in vitro* conditions.

Models of Latent infection

As processes and mechanisms pertaining to persistent infection may take long time to manifest in humans, various *in vitro* and *in vivo* models for *M. tuberculosis* latent infection have been proposed for study of processes and mechanisms related to the infection.

Most *in vitro* models exploit the stress conditions prevalent inside granuloma [13, 14], though none of them is able to simulate the *M. tuberculosis* latent infection fully. Owing to long generation time, highly infectious nature and requirement of BSL-3 (bio safety level) facilities for handling of *M. tuberculosis*, fast growing mycobacterial *sps.* (*M. smegmatis*) have been proposed as surrogate model organism for *M. tuberculosis* persistent infection. Thus, it is imperative to explore and establish alternate and better model organisms with shorter generation time for studies related to latent infection. The model organism should offer ease of working (in terms of lower bio-safety level and cost) and faster screening with existing protocols, so that it could be exploited for rapid screening of drugs and drug targets against LTBI (latent tuberculosis infection) and can also be exploited in deciphering various mechanisms pertaining to host pathogen interactions explicitly during latent TB infection. Thus, the present study was carried out to develop a holistic model simulating *M. tuberculosis* persistent infection conditions.

Objectives

1. Establishment and Validation of *M. fortuitum* Non-Replicating Persistence (NRP) model using hypoxia-induced stress

2. Molecular Validation and mechanism studies of *M. fortuitum* NRP model under hypoxia-induced stress

3. Establishment and Validation of *M. fortuitum* NRP model using acid-induced stress

Materials and Methods

Bacterial cultures and growth conditions

All experiments were carried on *E. coli* DH5α, *Mycobacterium aurum* A+ and *Mycobacterium fortuitum* ATCC 6841. *E. coli* DH5α cells were grown in Luria-Bertani broth or agar as required. *Mycobacterium* sps. were grown in MB7H9 broth, supplemented with 0.5% glycerol and 0.2% Tween80 [MBGT] and plated on Nutrient agar supplemented with 0.05%Tween80 [NAT]. All cultures were incubated at 37° C with constant shaking at 200 rpm to obtain actively growing aerobic cultures. Purity of the cultures as well as changes in morphology as attribute of bacilli under persistent form was checked by ZN (Ziehl-Neelsen) staining following standard protocols. Keeping in view the clumping nature of the genus, cultures were thoroughly vortexed prior to CFU (colony forming unit) determination in all the studies.

Cell viability under hypoxia-induced stress

Hypoxia-induced NRP model described by Dick et al. (1998) [13] was followed for study of growth kinetics and determination of cell viability of *M. fortuitum* and *M. aurum*. Cells were grown to an optical density of 0.4 - 0.7 (A_{600}), and methylene blue was added as an indicator of oxygen concentration to a final concentration of 1.5µg/mL and mixed. 7.5 mL of this actively growing culture was dispensed into 15 mL glass vials, thus maintaining HSR (head space ratio) of 0.5. Vials were capped with rubber septa, sealed with vacuum grease and incubated at 37°C. Oxygen depletion was monitored by decolorization of methylene blue. The viability of the culture was checked by CFU counts at regular intervals.

Validation of *M. fortuitum* as model organism under Hypoxia-induced stress

Persistent *M. tuberculosis* bacilli are shown to exhibit certain characteristic features such as changes in cell morphology, loss of acid fastness, physiological tolerance towards common anti-tubercular drugs, negligible metabolic activity and reactivation to an active infection (under favourable conditions). All these physiological features of persistent bacilli were studied to validate whether *M. fortuitum* model displays characteristic features of a persistent *M. tuberculosis* infection.

Changes or alterations in the cell morphology and staining characteristics were studied with ZN staining following a standard protocol. Changes in cell size and shape observed in staining were confirmed by TEM (transmission electron microscopy) of the samples.

For determination of physiological drug tolerance, MIC_{90} (minimum inhibitory concentration) was determined for actively replicating culture according to CLSI (Clinical & Laboratory Standards Institute) guidelines and same concentrations were applied to NRP state cultures. Percentage survival of the cells was compared when exposed to the drugs under replicating and persistent conditions.

Metabolic activity of the actively replicating cells and cultures subjected to hypoxia-induced NRP state was determined using pCDlux vector containing a luciferase reporter gene [15]. Vector containing the reporter gene was electroporated in *M. fortuitum* and expression of the reporter was then correlated to the metabolic state of the bacilli under actively replicating and NRP state.

To study reactivation, aliquot of cells under prolonged stress (1 month post anaerobiosis) were transferred to fresh media and incubated under shaking conditions at 200 rpm under aerated conditions at 37º C. CFU count was done at regular intervals to check growth and viability.

Validation of *M. fortuitum* NRP model by expression analysis

Construction of *Mycobacterium* - *E. coli* shuttle vector with *lacZ* reporter gene

A pMV206::*lacZ* vector was constructed to facilitate the cloning of upstream regulatory sequence of the genes, so as to assay their expression by β-galactosidase activity of the reporter gene. The structural gene coding for *lacZ* (β-galactosidase) was PCR amplified from pMV261*lacZ* [16]. This PCR amplified *lacZ* fragment was cloned into pMV206 Mycobacterium-*E. coli* shuttle vector [16]. The multiple cloning site upstream of *lacZ* reporter was retained to facilitate insertion of individual upstream regulatory sequence of *M. tuberculosis* genes.

β- Galactosidase assay for expression analysis of genes

Wild type and recombinant strains were analyzed and compared for β- galactosidase activity under aerobic and hypoxia induced NRP conditions using β- galactosidase assay [17], and the

reporter activity observed was expressed as Miller units. For β- galactosidase activity of cultures subjected to hypoxia-induced stress, the cultures were centrifuged and washed twice with PBST (PBS pH7.2, supplemented with 0.05% Tween80) to remove blue color due to addition of methylene blue, which could interfere with the absorbance values of the culture. The cells were vortexed to break clumps and resuspended in fresh LBGT medium containing lysozyme (3mg/mL). The suspension was incubated at 37 ºC for 1-2h to allow complete cell lysis. Following lysis, β- galactosidase assay was performed for actively replicating cultures, using standard protocol [17].

Standardization and Validation of *lacZ* expression for *M. smegmatis* latency conditions

Standardization of the β-galactosidase activity is required, as during persistent state number of cells per mL of the media decrease, and the cells enter a state of metabolic shut down resulting in an overall decrease in the expression of genes. For standardization of *lacZ* expression under replicating and hypoxia induced NRP conditions, a promoterless pMV206::lac*Z* vector was used as negative control; and β-galactosidase activity of the negative control was compared with that of a vector containing P*hsp60,* a constitutive mycobacterial promoter [18].

To validate whether the pMV206::*lacZ* vector could be used for expression analysis of genes under mycobacterial persistence conditions, β- galactosidase activity of two genes - *hsp*X (Rv2031c) [19] and *icl* (Rv0437) [20], known to be up regulated under hypoxia-induced persistent conditions was determined. Miller units obtained on expression analysis of different genes were normalized with respect to constitutive P*hsp*60 expression values obtained under NRP and actively replicating conditions.

Identification and sequence submission of *M. fortuitum* homologues of *M. tuberculosis* persistence genes

As *M. fortuitum* ATCC 6841 genome is not yet sequenced, thus in order to gain an insight into mechanism of persistence, a set of genes well established to be involved in *M. tuberculosis* persistence were used to design multiple sets of primers from conserved regions to amplify DNA sequences from *M. fortuitum.* Sequence of PCR amplified products were confirmed by sequencing. The longest sequences that showed maximum homology with corresponding *M. tuberculosis* genes were submitted to GenBank.

Five novel gene sequences were identified in *M. fortuitum*. These are probable homologues of *M. tuberculosis* genes, established to be involved in persistence. Additionally, to quantitate their expression during NRP state, primers were designed from *M. fortuitum* gene sequences. RNA was isolated from both actively replicating and NRP state for performing qRT-PCR. cDNA was synthesized using Verso cDNA synthesis kit with random hexamers and Verso RT enhancer (Thermo scientific) according to manufacturer's protocol. The amplification of the target sequence was detected using iScript one step RT PCR kit (Bio-Rad).

All reactions were run in triplicate. Relative fold changes were determined from the Cq values using the comparative Ct (ΔΔCt) method [21]. Gene expression studies were done under both -hypoxia-induced as well as acid-induced stress models.

Construction of antisense mutants of *Mficl, Mfsig*H and *Mfpca*A of *M. fortuitum*

Full length genes were PCR amplified from genomic DNA of *M. fortuitum* ATCC6841 as template using forward and reverse primers to which *Bam*HI sites were added at 5' ends. PCR amplified fragments were cloned into pGEM-Teasy TA cloning vector, followed by sequencing for confirmation of their sequence. TA constructs containing individual upstream sequences were digested with *Bam*HI, followed by purification using gel extraction kit. Purified inserts were ligated to pMV261 vector digested with *Bam*HI to obtain constructs containing genes inserted downstream to the constitutively expressing *hsp*60 promoter. These constructs were transformed into *E. coli* DH5α for amplification of the plasmid DNA and to check orientation of insert. The vectors were then checked for insertion of respective gene in forward (sense) or reverse (antisense) orientation downstream to the P*hsp*60 promoter in *M. tuberculosis* shuttle vector pMV261. The presence of full-length gene and its orientation in the construct was confirmed by restriction analysis and sequencing. Antisense constructs and the pMV261 vector without any insert (negative control) were then electroporated individually into *M. fortuitum* according to standard protocols. For confirmation of role of these antisense constructs in persistence, each recombinant strain with individual construct was subjected to hypoxia-induced NRP model and viability was determined by CFU counts at different time points.

Acid-Induced Stress Model

Cell viability under Acid-induced stress

Cells were subjected to acid-induced stress conditions, as acidic environment is reported to exist inside granuloma, the seat of latent bacilli. To study the growth kinetics under acid-induced stress, cultures were subjected to acidic stress at different pH. Seed culture was grown in 10 mL MBGT (pH6.5) to mid-exponential phase under aerobic conditions, with continuous shaking. Seed culture was passaged into fresh media, and the culture was overgrown for four days to achieve stationary phase to allow pre-adaption of the cultures. Stationary phase cells were washed twice to remove any traces of exhausted media. Washed cells were added to the flask containing 50mL of MB7H9 media at pH of 6.5, 5.5, 4.5, 3.5, 2.5 and 1.5. Density of the cells (w/v) transferred to acidic media was maintained, and was similar to that of stationary phase culture. To study the importance of pre-adaptation, an aliquot of 50 mL of culture was subjected to gradual pH change from the range of 6.5 to 1.5. For this after every 24 hours cells were washed thrice with subsequent lower pH media and resuspended in 50mL of the same media. 0.1N HCl was used to adjust pH of the media wherever required.

Hallmark features of persistent bacilli such as changes in cell morphology, loss of acid fastness, physiological tolerance towards common anti-tubercular drugs, negligible metabolic activity, reactivation, and expression of *M. fortuitum* genes were studied in similar manner as described above for hypoxia-induced NRP model.

Results and Discussion

M. aurum and *M. fortuitum* as model organism to study *M. tuberculosis* persistent infection

Literature survey was done to shortlist rapidly growing, mild pathogenic mycobacterial species. Mild pathogenic and rapidly growing species offered advantage with ease of handling in lower biosafety level and faster growth respectively. Phylogenetic proximity and genome homology were further compared to further shortlist number of organisms for the study. Thus, on the basis of generation time, ease of handling (non requirement of BSL3), and homology - *M. aurum* and *M. fortuitum* were selected for development of a surrogate model mimicking *M. tuberculosis* persistent infection.

Mycobacterium aurum is unable to survive in hypoxia-induced NRP model

Among the known non-pathogenic fast growing mycobacteria, *M. aurum* is reported to be closer to *M. tuberculosis* in terms of mycolate components and presence of cyclopropane ring [22]. Pigmentation is considered to be an added advantage of using *M. aurum* so that contaminants could be easily marked off. The bacilli also possess a high level of resemblance to *M. tuberculosis* in terms of susceptibility profile against cell wall inhibitors. *M. aurum* has been recently used as integrated surrogate model for screening of drugs against *M. tuberculosis* [23]. The ability of intracellular survival inside macrophages [24, 25] parallel to *M. tuberculosis* is another advantage to utilize *M. aurum* for development of a surrogate model to simulating *M. tuberculosis* latency.

M. aurum cells were subjected to granuloma specific stress conditions of hypoxia and acidic pH induced stress. *M. aurum* cells failed to survive for extended periods of time when subjected to hypoxia induced stress conditions **(Fig. 1)**. Sealed vials containing *M. aurum* cultures showed fainting of methylene blue color after day 6, and complete discoloration was observed between days 10 to 12 indicating hypoxic environment. *M. aurum* cells showed viability up to day 16 under gradual oxygen depletion conditions. A sharp decline in CFU count was observed 16 days post sealing of the vials and no CFU count was observed after 20 days. Cell viability was further checked till 45 days post sealing of vials with no reversal of cell viability (data shown only till 30 days post sealing and 20 days after hypoxia). Thus, as

M. aurum cells could not survive under hypoxia-induced stress conditions, it cannot be used for development of a model to mimic *M. tuberculosis* persistent infection.

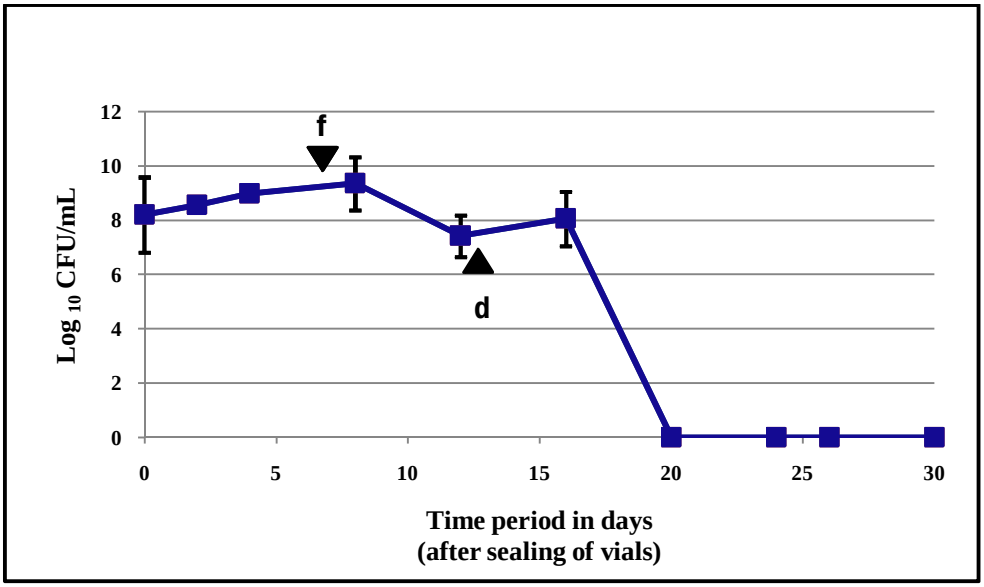

Fig. 1 Growth and survival of *M. aurum* cells under hypoxia induced NRP model: *M. aurum* cells were subjected to hypoxia-induced NRP model. **'f'** indicates fading of blue color and **'d'** indicates complete discoloration of methylene blue color indicating hypoxic conditions. *M. aurum* cells were viable upto day 16, sharp decline in CFU was observed 16 day post sealing. Cells did not show constant CFU or survival for extended periods of time under hypoxic condition a characteristic of latent infection.

Mycobacterium fortuitum enters Non-Replicating Persistence state on exposure to hypoxia-induced stress condition

To study the behaviour of *M. fortuitum* under hypoxia-induced stress, viability of *M. fortuitum* was monitored during hypoxia. Continuous shaking was avoided to maintain gradual oxygen diffusion from air space to medium. Blue color of methylene blue indicator dye started to faint during first week and complete discoloration was observed between first and second week. The culture showed a constantly maintained CFU for extended periods of time of ten weeks **(Fig. 2)**. Non-replicating persistence associated latency is reported both in slow growing [26] as well as rapidly growing mycobacteria [13]. Wayne and co-workers have reported that under hypoxia-induced stress condition *M. tuberculosis* survives for extended periods of time. In our study, we also observed that *M. fortuitum* exhibited NRP state when subjected to hypoxia-induced stress condition, which is in accordance with that of *M. tuberculosis.*

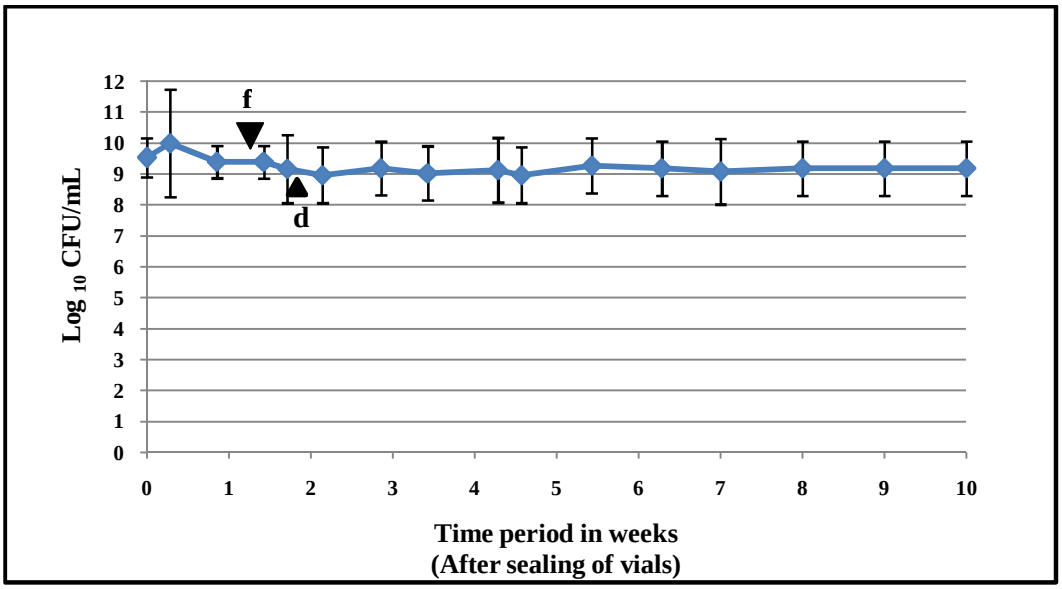

Fig. 2 Growth and survival of *M. fortuitum* cells under hypoxia induced NRP model. Log_{10} CFU/mL as a function of time is shown in this graph. Cells were grown to logarithmic phase and incubated in sealed tubes. CFU was monitored for 10 weeks, cells showed survival and constant CFU (NRP) for extended period of time. 'f' indicates fading and 'd' indicated complete decolorization [Data shows a mean of 3 experiments with S.D in error bars].

Mycobacterium fortuitum as model to study *Mycobacterium tuberculosis* persistent infection

(a) Changes in cell morphology and cell wall thickening

During advancement to NRP state, the bacilli are shown to exhibit morphological changes in cell shape, size and display altered staining characteristics. Findings pertaining to morphological changes in our study are equivalent to those as reported earlier [27]. When subjected to hypoxia stress induced NRP; the bacilli showed reduced acid fast staining, they showed barred appearance and were stouter in comparison to the actively replicating bacilli **(Fig. 3)**.

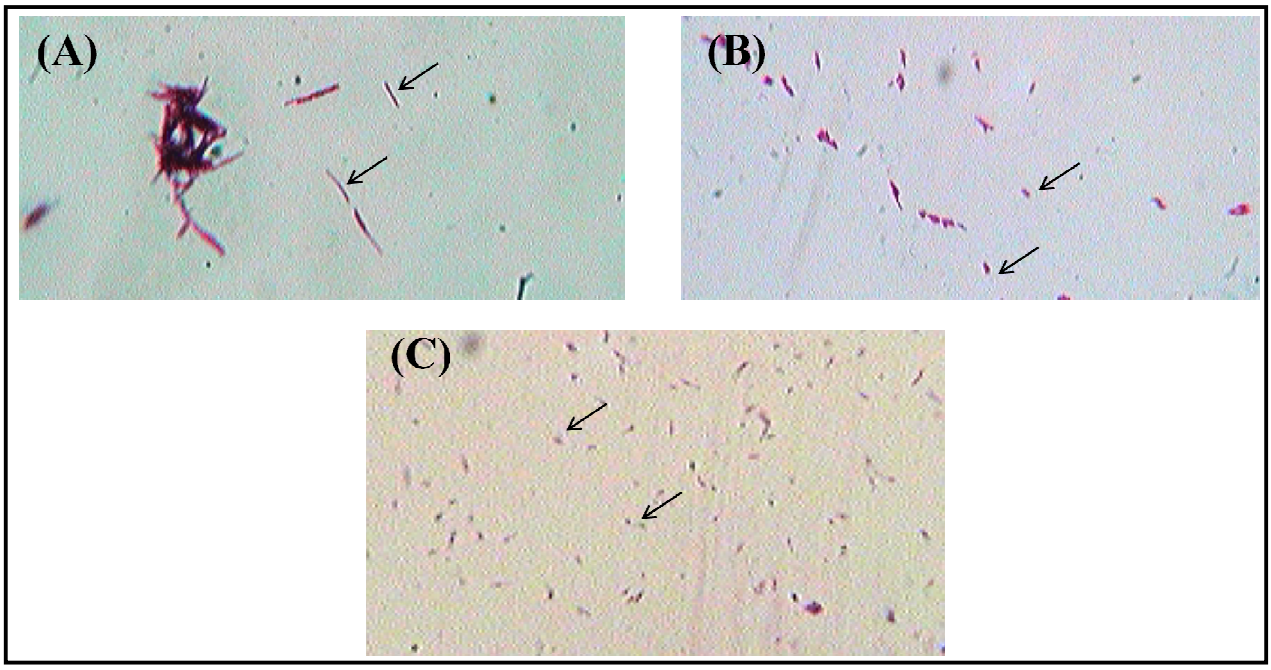

Fig. 3 ZN staining of cultures under actively replicating and NRP state [1000X] Oil immersion. *M. fortuitum* cells were stained using Ziehl-Neelsen method for acid fast bacteria. **(A)** Actively replicating cells showed normal morphology and staining characteristics (bacilli stained dark pink in color and longer in size) in comparison to **(B)** Non-Replicating persistent state-I cells exhibiting altered staining characteristics (bacilli stained light pink in color and shorter size) **(C)** NRP II state cells stained weak acid fast and appeared shorter in size.

This suggests that the bacilli have undergone modifications related to expression of genes encoding for cell wall synthesis and typically related to mycolic acids as composition and integrity of mycolic acids form major thrust of acid fastness exhibited by the genus mycobacterium. Morphological differences observed in cell shape and sizes were further confirmed by transmission electron microscopy, where significant shortening of size and thickening of cell wall were observed.

(b) Lowered metabolic activity

Cells under NRP state showed negligible metabolic activity (less than 500 RLU [relative light units] in comparison to the actively replicating cells (50000-80000 RLU) indicating that under persistent state there is slowdown of cell metabolic activity (figure not shown). As cells are in dormant or sleeping form requirement of energy is minimal due to inhibition of various cell activity.

(c) Physiological tolerance towards anti-tubercular drugs

NRP bacteria are documented to exhibit resistance towards commonly used antimycobacterials [26]. These drugs act either on cell wall, protein synthesis or on enzymes for DNA synthesis. Since most transcription and translation machinery is shutdown, it leads to shift in MIC (minimum inhibitory concentration) due to tolerance developed by the NRP bacteria. Cells under NRP exhibited tolerance towards commonly used antimycobacterials **(Table 1)**. These findings are in agreement to those reported in case of pathogenic as well as opportunistic pathogens which have been shown to persist when subjected to stress conditions. When the NRP state cells are subjected to fresh media, the reactivated state cells show a reversal to sensitivity towards antimycobacterials drugs, similar to actively replicating cultures. Hence shift in MIC for NRP state bacteria can be termed as 'physiological tolerance'.

Table 1: Antibiotic susceptibility of persistent *M. fortuitum* was determined for rifampicin, isoniazid and ethambutol. MIC was determined for actively replicating state and same concentrations were applied on NRP state cultures. For NRP stage cultures antibiotics were added 10 days post anaerobiosis through rubber septum and CFU was determined. Bacilli under NRP state exhibited antibiotic tolerance. **S** = sensitive < 25%; **T**= tolerant > 75%

Antibiotic (MIC_{90})	**Actively Replicating State**	**NRP State**
Ethambutol (20µg/mL)	S	T
Isoniazid (32µg/mL)	S	T
Rifampicin (16µg/mL)	S	T

(d) Reactivation of persistent bacteria after removal of stress condition:

When an aliquot of cells under prolonged stress (1 month post anaerobiosis) was transferred to fresh media and incubated under shaking conditions at 200 rpm under aerated conditions at 37º C the bacteria showed an extended lag phase and thereafter entered a normal replication growth curve **(Fig. 4)**.

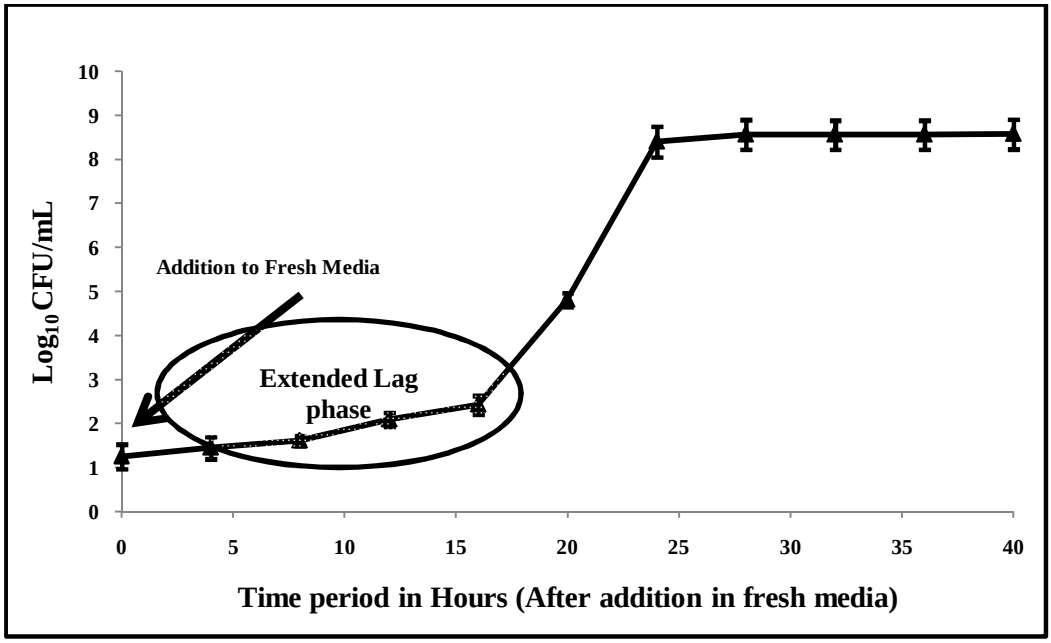

Fig. 4 Reactivation of NRP state bacteria on transfer to fresh media. One month old NRP stage culture of *M. fortuitum* showed reactivation to normally actively replicating state on transfer to fresh media. Bacteria during reactivation showed a comparatively longer lag phase and then entered logarithmic phase to follow a normal growth curve.

pMV206::*lacZ* vector for Validation of *M. fortuitum* as model by expression analysis

A pMV206::*lacZ* shuttle vector was constructed containing *E. coli* (*col*E1) and mycobacterial (oriM) origins of replication, kanamycin resistance gene (Km^r) as selection marker, and a *lacZ* reporter gene. Presence of a multiple cloning site upstream to the *lacZ* reporter gene facilitates cloning of upstream regulatory sequence of the genes. Size of the vector was estimated to be 7610bp. The vector was used for expression analysis of genes under actively replicating and hypoxia-induced NRP model of persistent infection.

M. fortuitum containing promoterless vector showed *lacZ* expression values similar to that of wild type *M. fortuitum* strain, suggesting negligible basal activity of the reporter. P*hsp60* showed positive and significant expression in comparison to *M. fortuitum* containing promoterless vector and wild type *M. fortuitum* under actively replicating and hypoxia induced persistent conditions, indicating that the vector is functional under both conditions. Promoters from genes known to be upregulated under *M. tuberculosis* persistent- P*hspX* (alpha crystalline) and P*icl* (isocitrate lyase) showed increased expression of 40 fold and 28 fold respectively, under hypoxia induced persistence in comparison to their actively replicating state, validating utility of the vector under NRP conditions. It is well recognized that during achievement of NRP; normal metabolism of the bacilli shutdown and it utilizes alternate secondary metabolic pathways for survival. *Icl* (isocitrate lyase) is acknowledged for being an important enzyme during gradual shift to NRP [7]. Alpha crystalline is responsible for cell wall thickening during NRP state [28]. This up regulation of '*acr'* can also be correlated with cell wall thickening observed in electron microscopy images. The cell wall thickening may also elucidate drug tolerance portrayed by bacilli during NRP, which in a way may shield the bacilli from various antimycobacterial drugs during hypoxia stress.

Identification of *M. fortuitum* genes involved in Hypoxia-induced Non Replicating Persistence

M. fortuitum showed constant CFU and other physiological characteristics of persistent bacilli. To make an insight into mechanism of persistence in *M. fortuitum*, *M. fortuitum* homologues of genes involved in persistence of *M. tuberculosis* were identified. Five novel genes were submitted to GenBank (**Table 2**).

Table-2 Novel gene sequences submitted to GenBank and their percent identity with *M. tuberculosis*

Gene	GenBank ID	Percent identity with corresponding *M. tuberculosis* gene/protein	
		Gene	Protein
Mficl	1749100 [KM275229]	86.48	90.65
*Mfpca*A	1750033 [KM282163]	68.98	60.82

MfsigH	1750394 [KM282166]	78.36	87.26
*Mfnar*X	1750034 [KM282164]	80.18	86.94
*Mfnar*K2	1750079 [KM282165]	69.96	68.94

These sequences are hypothetical *M. fortuitum* homologues of *M. tuberculosis* genes isocitrate lyase (*icl*), sigma factor *sigH*, *pcaA*, nitrate reductase *narX* and nitrate/nitrite transporter *narK2* respectively. These homologues showed significant upregulation under hypoxia induced NRP state as quantitated by qRT-PCR, which indicates their probable role in of non-replicating persistent state of *M. fortuitum* **(Fig. 5)**. *Icl* is well documented to play role in *M. tuberculosis* persistence [7]. *pca*A also plays an important role in persistence both in BCG and *M. tuberculosis*. *pca*A mutants fail to persist in mice. *sig*H is also documented to play vital role in gene regulation that allows the bacteria to acclimatize and endure during different stress conditions that organism is subjected to likely encounter during infection. Studies also confirm that *M. tuberculosis* alternate sigma factor, *sig*H, is induced after heat, redox and acid stress and phagocytosis and mutant is unable to persist [7]. *narX* is the first gene that is transcriptionally active during dormancy in *M. tuberculosis* and can serve as important marker during dormancy [29]. All five genes showed significant upregulation in our study indicates that these genes might play some role during hypoxia induced stress.

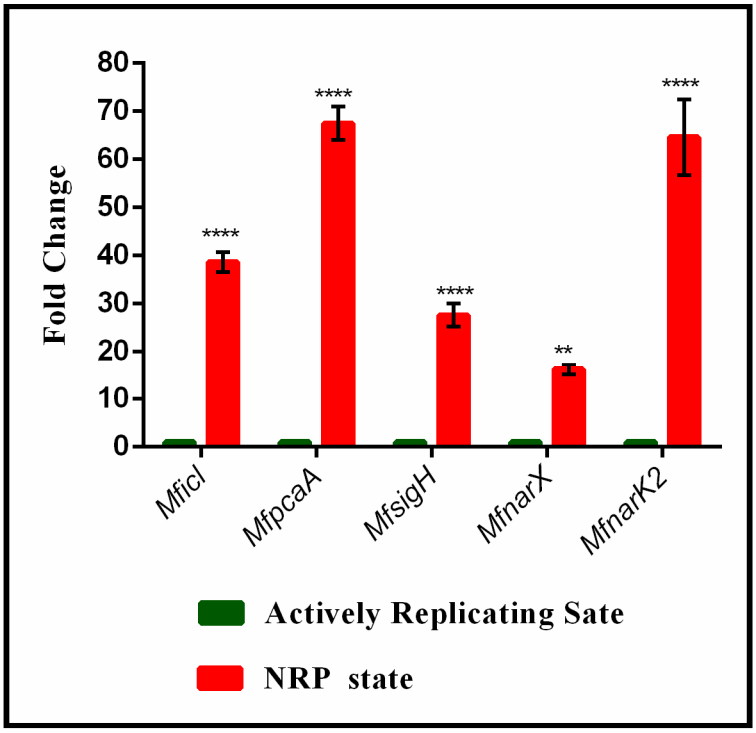

Fig. 5 Expression analysis of genes by qRT-PCR under Hypoxia-induced NRP model Gene expression determined by qRT-PCR. 16S of *M. fortuitum* was taken as internal control and expression of other genes was normalized. Graph shows induced fold change in NRP stage as compared to actively replicating (AR) state. Upregulation of all five hypothetical genes predicted to be homologues of *M. tuberculosis*, indicates that they might play some role during hypoxia induced stress in *M. fortuitum*.

Antisense knock-out of hypothetical *Mficl, MfsigH* and *MfpcaA* of *M. fortuitum*

Constructs of genes carrying insertion in reverse (antisense) direction downstream of the P*hsp*60 promoter were selected for the study. *In vitro* analysis was done under hypoxia-induced NRP model of persistence to check expression level of antisense constructs in comparison to vector control and wild type strains. Survival under hypoxia induced stress model was determined by CFU counts at different time points (**Fig. 6**).

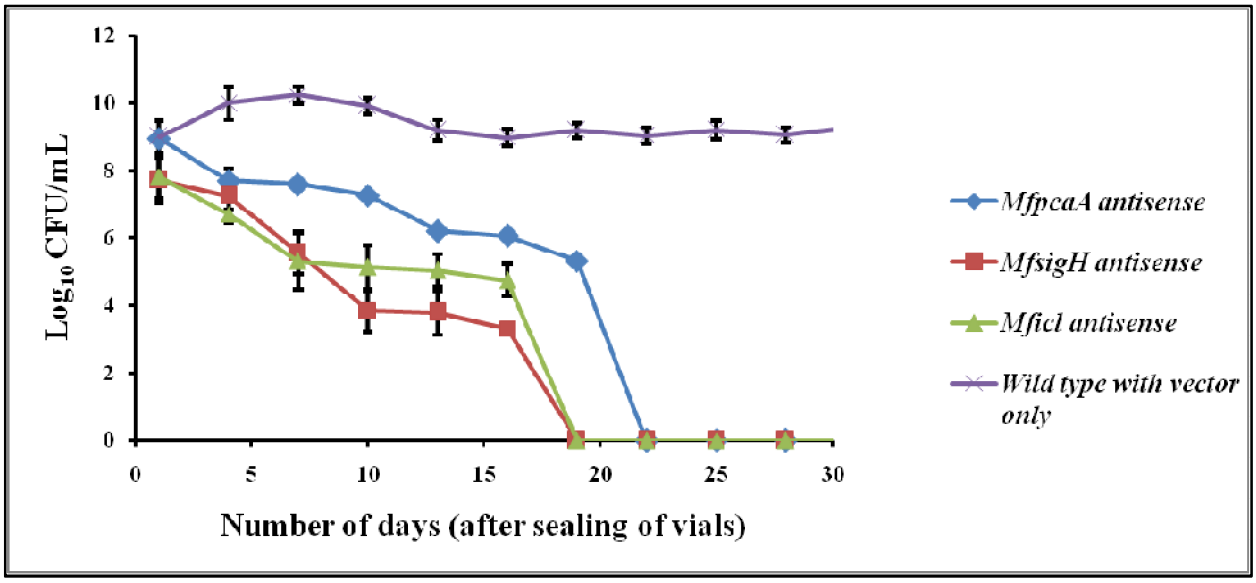

Fig. 6 Survival of recombinant *M. fortuitum* antisense knock out mutants. Wild-type *M. fortuitum* strain and *M. fortuitum* transformed with pMV261 antisense knockout of *Mfpca*A, *Mfsig*H and *Mficl* were checked for survival under persistence model (hypoxia-induced NRP). CFU as function of time is shown in the graph. All three recombinant strains with antisense knockout constructs showed reduced survival and failed to persist for longer periods of time in comparison to vector control.

MfpcaA antisense strain of *M. fortuitum* failed to survive for longer periods under hypoxia induced persistence model indicating its role in persistence of *M. fortuitum. M. tuberculosis* Δ*pcaA* strain is also reported to exhibit altered cell surface properties and is involved in persistence. *Mficl* antisense strain of *M. fortuitum* showed survival for about two weeks after which sharp decline in CFU was observed. *Isocitrate lyase* is enzyme essential for the

metabolism of fatty acids. Disruption of the *M. tuberculosis icl* gene attenuated bacterial persistence and virulence in immune-competent mice. Similarly, *MfsigH* antisense mutant of *M. fortuitum* failed to survive in hypoxia-induced NRP model. The expression of *M. tuberculosis sigH* is induced in response to heat shock and oxidative stress, and following uptake by macrophages. Antisense constructs of overlapping genes *Mfnar*X and *Mfnar*K2 could not be constructed.

M. fortuitum acid-induced stress based model for *Mycobacterium tuberculosis* persistent infection

Multiple studies have reported survival of *M. tuberculosis* under acidic stress, and correlated the altered gene expression during acid-induced stress to persistent state of *M. tuberculosis* [30, 31]. Acid-induced stress has been used as a one of the pivotal conditions in multiple stress model for *M. tuberculosis* [32]. Thus, *M. fortuitum* cells were subjected to acid-induced stress conditions. *M. aurum* cells did not show survival under acid-induced stress condition, and the cells were viable only for short duration of 4 days (data not shown). During the study, cells lost pigmentation and pale colonies were observed, but retained their acid fast nature on ZN staining.

Thus, non survival of *M. aurum* cells under acid-induced stress condition related to *M. tuberculosis* latency in addition to hypoxia-induced stress conditions further confirms that *M. aurum* is not a suitable model organism to study *M. tuberculosis* latent infection.

On the other hand *M. fortuitum* cells showed a constantly maintained CFU under acidic pH stress of 5.5, 4.5 and 3.5 till 10 weeks of observation period **(Fig. 7)**. Below pH 3.5 instant cell death was observed, hence all further studies were carried under 5.5, 4.5 and 3.5 pH conditions.

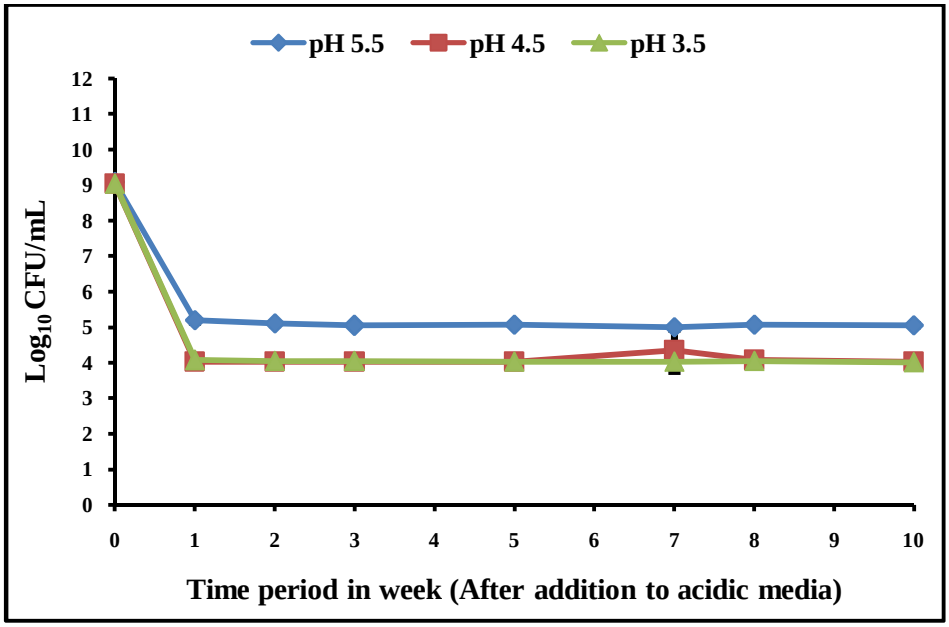

Fig. 7 Growth and survival of *M. fortuitum cells* under acid-induced stress at pH 5.5, pH 4.5 and pH 3.5. Log_{10} CFU/mL as a function of time is shown in this graph. *M. fortuitum* cells showed constant CFU for ten weeks at acidic pH.

In cultures where gradual pH change was done the culture completely fail to survive, thus indicating that pre-adaptation is important for achieving persistent state of the bacilli. Our results suggest that acidification of macrophage or granuloma may provide some initial signals that are required for survival during persistence of *M. tuberculosis* inside the host.

Validation of *M. fortuitum* acid-stress based model for *Mycobacterium tuberculosis* persistent infection

(a) Changes in cell morphology and cell wall thickening

There are well documented evidences of change in cell shape, size and loss of acid-fastness during NRP state. *M. fortuitum* cells showed changes in cell shape, size and acid fastness when subjected to acidic stress conditions. The bacilli were shorter in size and retained less stain as compared to that of actively replicating state. Bacteria showing constant CFU also showed changes in acid fastness and cell size **(Fig.8)**. Images from electron microscopy studies also confirmed shortening of size of bacteria showing persistence.

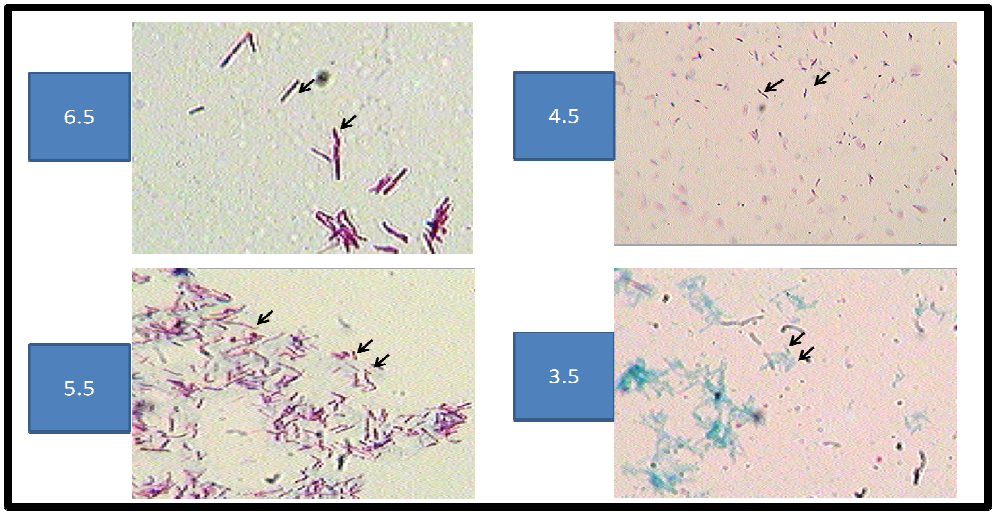

Fig. 8 Ziehl-Neelsen staining of the bacilli under normal and acid-induced stress conditions. Persistent *M. fortuitum* cells showing changes in cell shape, reduction in size and loss of acid fastness (pH5.5, 4.5, 3.5) in comparison to actively replicating cells (pH 6.5).

(b) Lowered metabolic activity

Cells under acid stress induced NRP state showed negligible metabolic activity in comparison to the actively replicating cells indicating slowdown of cell metabolic activity that under persistent state (figure not shown).

Physiological tolerance towards anti-tubercular drugs

Antibiotic susceptibility of *M. fortuitum* cells subjected to acid-induced stress conditions was determined for rifampicin, isoniazid and ethambutol. Persistent *M. fortuitum* cells showed physiological tolerance to antitubercular drugs under acid-induced stress conditions **(Table 3)**. These findings are similar to those observed under hypoxia-induced stress.

Table 3: Antibiotic susceptibility of *M. fortuitum cells* under actively replicating and acid-induced stress

Antibiotic (MIC_{90})	**Actively Replicating culture (pH 6.5)**	**Culture under acid-induced stress**		
		pH 5.5	**pH 4.5**	**pH 3.5**
Ethambutol (20µg/mL)	S	T	T	T
Isoniazid (32µg/mL)	S	T	T	T

Rifampicin (16µg/mL)	S	T	T	T

S = sensitive < 25%; **R**= resistant > 75%

(d) Reactivation to normal replication cycle on transfer to fresh media

As in case of hypoxia-induced stress *M. fortuitum* cells showed reactivation to normal actively replicating cells and entered log phase of growth after a longer lag phase (about 14-16h) **(Fig. 9)**. These finding are in agreement to earlier reports where NRP state bacilli resume normal growth on withdrawal of stress conditions.

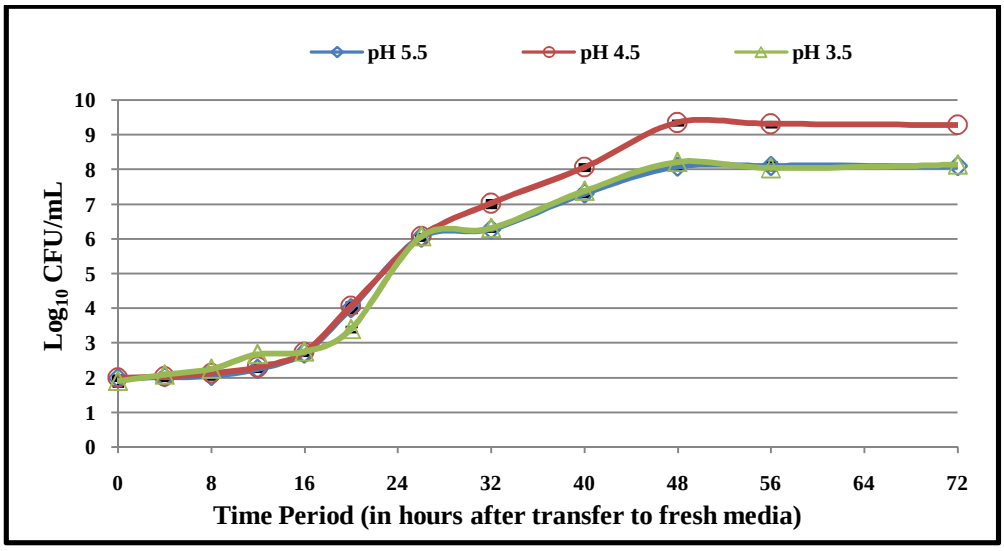

Fig. 9 Reactivation of cells from NRP state to normal growth phase after addition to fresh media. One month-old acid stressed culture of *M. fortuitum* was transferred to fresh media (pH 6.5) and incubated for growth under aerobic conditions. The bacteria showed an extended lag phase and then enterer log phase to follow a normal growth curve. Data shows mean of three independent experiments with S.D in error bars.

Expression analysis by qRT-PCR

All genes (*Mf*icl, *Mf*pcaA, *MsigH*, *Mf*narX and *Mf*narK2) showed significant upregulation at pH 5.5 and 4.5 and 3.5 **(Fig. 10)**. Interestingly, *Mfsig*H showed significant up-regulation at all three different conditions and increase was directly proportional to increase in stress. From this expression data it can be hypothesised that an acidic pH of around 4.5 might be acting as signal for shift to a persistent state as maximum upregulation in all genes was observed at this pH.

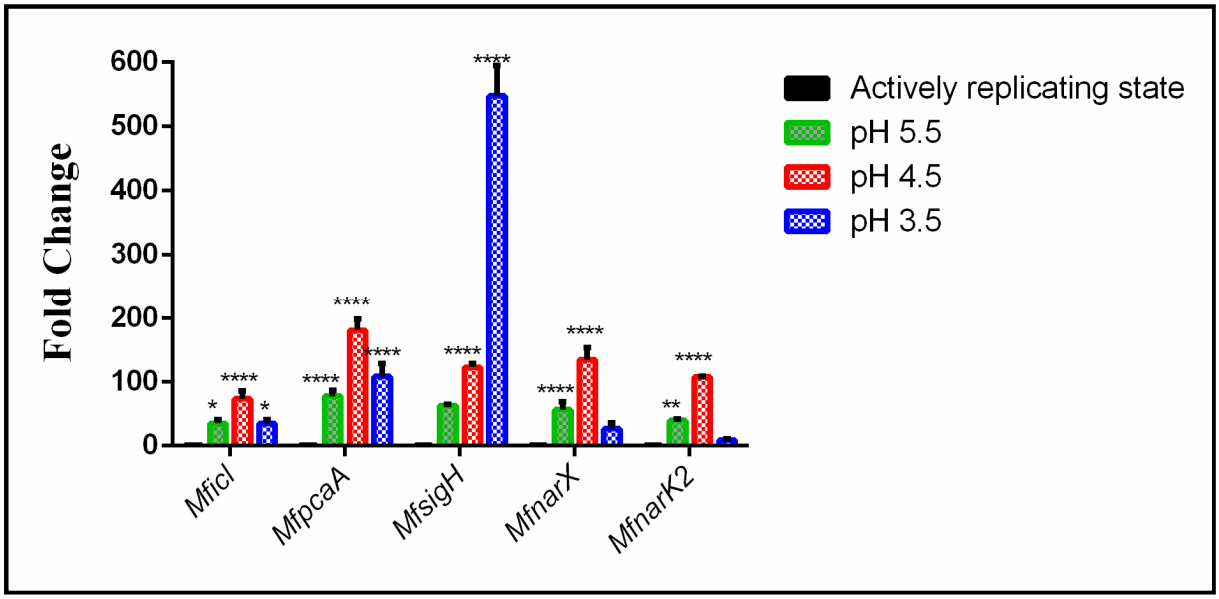

Fig. 10 Expression analysis of genes by qRT-PCR under acid-induced stress. Gene expression was determined by qRT-PCR. 16S of *M. fortuitum* was taken as internal control and expression of other genes was normalized. Graph shows induced fold change under acidic stress at three different pH values as compared to actively replicating (AR) state. All five gens showed upregulation under acid induced stress.

From this study related to acid-induced stress it can be concluded that acid-induced stess can be used as a physiological stress condition to develop model for *M. tuberculosis* persistent infection. Though this is a primary conclusion with *M. fortuitum* and requires further validation in *M. tuberculosis.*

Conclusion

Success of *M. tuberculosis* lies in its ability to persist in human body for extended periods of time without causing clinical disease. Effective control of TB is largely held back by persistent form of infection. Outcomes from studies exploiting currently available models of latent infection have been unable to yield any conclusive solutions pertaining to latency or reactivation. Establishment of holistic model which is completely able to simulate the conditions of persistent infection will help in rapid and swift screening of drugs and drug targets specific to latent state of infection. Our study showed that *M. aurum* could not survive under hypoxia-induced and acid-induced conditions related to *M. tuberculosis* latency and hence, cannot be used as *in vitro* model to study *M. tuberculosis* latent infection. On the other hand, *M. fortuitum* can be utilized as model, as bacilli maintained constant CFU when subjected to hypoxia induced stress. Other cardinal changes pertaining to NRP state were also evident. Additionally, up-regulation of promoters of *M. tuberculosis* genes involved in persistence was also observed. *M. fortuitum* may have a similar mechanism of persistence as homologues of five genes involved in *M. tuberculosis* persistence were identified in *M. fortuitum.* All genes showed significant up-regulation during persistence indicating a similar mechanism of persistence. Antisense knockout strains of 3 genes showed inability to survive under hypoxia stress induced conditions, suggesting their involvement and requirement for *in vitro* survival during persistence. *M. fortuitum* also showed persistence under acid-induced stress by maintaining constant CFU and other associated physiological changes. Thus, we have developed and validated an *M. fortuitum in vitro* model for *M. tuberculosis* persistence which can be used for faster screening of potential drugs and drug targets. It might also be used to explore the mechanism of persistence and reactivation in *M. fortuitum*, correlates of which can be found in *M. tuberculosis* and other mycobacterial species.

Publications

Journal publications

1. **Shivani Sood**, Satinder Kaur, Rahul Shrivastava (2015). A *lacZ* Reporter-Based Strategy for Rapid Expression Analysis and Target Validation of *Mycobacterium tuberculosis* Latent Infection Genes. Current Microbiology, 72(2), 213-219. (10.1007/s00284-015-0942-3).
2. **Shivani Sood**, Anant Yadav, Rahul Shrivastava. (2016). *Mycobacterium aurum* is Unable to Survive *Mycobacterium tuberculosis* Latency Associated Stress Conditions: Implications as Non-suitable Model Organism. Indian Journal of Microbiology, 56(2), 198-204.
3. **Shivani Sood**, Poonam, Rahul Shrivastava. *Mycobacterium fortuitum* as surrogate in acid stress based *in-vitro* model for *Mycobacterium tuberculosis* persistent infection. (Communicated).
4. **Shivani Sood,** Rahul Shrivastava. *Mycobacterium fortuitum* shows a conserved mechanism of Non-replicating Persistence on exposure to hypoxia induced stress (Under draft).

GenBank Submissions

1. **Shivani Sood**, Varun Jaiswal, Poonam Katoch, Sandeep Kumar Sharma and Rahul Shrivastava *M. fortuitum* isocitrate lyase, a homologue of *M. tuberculosis* H37Rv isocitrate lyase. [BankIt 1749100 Sequence 1 KM275229]
2. **Shivani Sood**, Varun Jaiswal, Poonam Katoch and Rahul Shrivastava. *M. fortuitum* homologue of *M. tuberculosis* sigma factor SigH. [BankIt 1750394 Sequence 1 KM282166]
3. **Shivani Sood**, Varun Jaiswal, Poonam Katoch and Rahul Shrivastava. *M. fortuitum* pcaA, a homologue of *M. tuberculosis* H37Rv pcaA. [BankIt 1750033 Sequence 1 KM282163]
4. **Shivani Sood**, Varun Jaiswal, Poonam Katoch and Rahul Shrivastava. *M. fortuitum* nitrate reductase narX, a homologue of *M. tuberculosis* H37Rv nitrate reductase narX. [BankIt 1750034 Sequence 1 KM282164]
5. **Shivani Sood**, Poonam Katoch, Varun Jaiswal and Rahul Shrivastava. *M. fortuitum* nitrate/nitrite transporter narK2, a homologue of *M. tuberculosis* H37Rv nitrate/nitrite transporter narK2. [BankIt 1750079 Sequence 1 KM282165]

Conference Publications

1**. Shivani Sood**, Poonam Katoch, Rahul Shrivastava (2015). A conserved molecular mechanism of adaptation on exposure to acidic condition shown by fast growing pathogenic mycobacteria *Mycobacterium fortuitum*. Abstract selected for e-poster presentation at 25th European Congress of Clinical Microbiology and Infectious Diseases, Copenhagen, Denmark. [25-28 April 2015]

2. Rahul Shrivastava, **Shivani Sood**, Poonam, Jitendraa Vashistt (2014). Reporter based two-pronged strategy: target validation and rapid drug screening against persistent mycobacteria. XXXVIII All India Cell Biology Conference and International Symposium on "Cellular Responses to Drugs", CDRI Lucknow, India. [10-12 Dec, 2014]

3. **Shivani Sood**, Anant Yadav, Poonam, Rahul Shrivastava (2014) *M. fortuitum* persistence on exposure to granuloma specific conditions: Implications for a surrogate model. Proceedings of the International conference on Cellular and Molecular Mechanisms of Disease Processes, Srinagar, Jammu & Kashmir, India. [13-16 April, 2014]

4. **Shivani Sood**, Raj Kumar Tiwari, Neeraj Mahindroo, Rahul Shrivastava (2013) Establishment of Rapidly Growing Mycobacteria (RGM) as drug screening model for *M. tuberculosis* latent infection. Proceedings of the National conference on Research Trends in Drug Development: Exploration of Medicinal & Aromatic Plants Shoolini University, Solan H.P., India. [20-21 June, 2013]

5. **Shivani Sood**, Sree Krishna Chanumolu, Chittaranjan Rout, Rahul Shrivastava (2012) Identification of Mycobacterial genes involved in Persistence as Potential Drug Targets. Proceedings of the Conference on NexGen Biotechnology: Amalgamating Science & Technology [UIET, Kurukshetra University, Kurukshetra, India. [23-24 Nov, 2012]

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